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Tumor Necrosis Factor-α Promoter Variant 2 (*TNF2*) Is Associated With Pre-term Delivery, Infant Mortality, and Malaria Morbidity in Western Kenya: Asembo Bay Cohort Project IX

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A polymorphism in the promoter region of the tumor necrosis factor-α (TNF-α) gene, with a guanine to adenine nucleotide change at position –308, *TNF2* is associated with increased TNF-α production. *TNF2* homozygotes have a higher risk of severe disease and/or death due to cerebral malaria and other infectious diseases. We investigated the impact of this allele on malaria morbidity and mortality in young children who participated in an immuno-epidemiologic cohort study of malaria in an area of intense perennial *Plasmodium falciparum* transmission in western Kenya. A total of 1,048 children were genotyped. Poisson regression and Cox proportional hazards models were used to determine the relationship between TNF-308 variants and morbidity and mortality. The gene frequencies of the *TNF1* and *TNF2* alleles were 0.90 and 0.10, respectively. *TNF2* homozygosity was associated with pre-term birth when compared with *TNF1* homozygotes [relative risk (RR) 7.3, 95% CI, 2.85–18.9, $P = 0.002$) and heterozygotes (RR 6.7, 95% CI 2.0–23.0, $P = 0.008$). Among children born prema-

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turely, the *TNF2* allele was significantly associated with a higher risk of death in infancy compared with *TNF1* (RR 7.47, 95% CI 2.36–23.6). The risk of death was higher among *TNF2* homozygotes than among heterozygotes. The *TNF2* allele was significantly associated with high density *P. falciparum* parasitemia (RR 1.11, 95% CI 1.0–1.24). Among low birth weight children, the *TNF2* allele was associated with severe anemia (RR 2.16, 95% CI 1.17–4.01) and showed a trend toward a risk for severe malaria anemia (RR 1.99, 95% CI 0.89–4.46). These data suggest that *TNF2* is a risk factor for pre-term birth and early childhood mortality and malaria morbidity in children in this region. Further understanding of the pathogenic mechanisms underlying this association is required. Genet. Epidemiol. 21:201–211, 2001.

Key words: polymorphism; malaria; premature birth

INTRODUCTION

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine known to be essential in the control of many intracellular infectious agents in humans [Beutler and Grau, 1993]. Elevated levels of TNF-α have, however, been implicated in the pathogenesis of infectious diseases such as malaria [Kwiatkowski et al., 1990], meningococcal disease [Westendorp et al., 1995; Riordan et al., 1996], schistosomiasis [Amiri et al., 1992], and some autoimmune diseases [Wilson et al., 1995]. In addition, several studies have shown that increased TNF-α levels in placental and fetal tissues are associated with spontaneous abortions and pre-term delivery [Girardin et al., 1990; Shaarawy et al., 1997; Arntzen et al., 1998; Romero et al., 1998; Wenstrom et al., 1998].

A single nucleotide polymorphism from guanine (G) in the normal *TNF1* allele to adenine (A) in the *TNF2* variant at position –308 relative to the transcription start site of the TNF-α gene is associated with increased TNF-α production [Wilson et al., 1997]. The *TNF2* allele is associated with lepromatous leprosy [Roy et al., 1997], scarring trachoma infections [Conway et al., 1997], mucocutaneous leishmaniasis [Cabrera et al., 1995], and a higher risk of death in meningococcal disease [Nadel et al., 1996]. Homozygosity for this rare *TNF2* allele has been associated with a fourfold increased risk of cerebral malaria and seven times greater likelihood of death after a cerebral malaria attack [McGuire et al., 1994]. In another study, *TNF2* was found to be associated with severe malaria outcomes (multiple-organ dysfunction syndrome) and impaired consciousness due to infectious diseases other than malaria [Wattavidanage et al., 1999]. Although these studies identified *TNF2* as a risk factor for morbidity and mortality after infection with different pathogens, the overall impact of this allele on child survival is not known. Furthermore, the impact of this allele on the severity of malaria is unknown in areas with intense malaria transmission such as western Kenya, where severe anemia and not cerebral malaria is the most common manifestation of severe malaria. Taking advantage of a longitudinal birth cohort (Asembo Bay Cohort) in western Kenya, we retrospectively determined the relationship between the *TNF2* allele and all-cause mortality and malaria-specific morbidity. Here, we report an association of *TNF2* with birth outcome and infant survival. In addition, the impact of this allele on malaria-associated morbidity in this population is described.

MATERIALS AND METHODS

This study was a retrospective investigation using DNA from participants enrolled in a mother-infant cohort study, the Asembo Bay Cohort Project (ABCP) conducted in western Kenya. More than 95% of the residents in this area belong to Luo ethnic group. The methodology and cohort design of the ABCP were described elsewhere [Bloland et al., 1999]. In the study, infants were followed up from birth to 5 years of life with mortality and monthly malaria-associated morbidity data collected. Briefly, pregnant women identified through monthly census were enrolled after informed consent was obtained. Within 24 hours of delivery, the infant's birth weight was obtained, and gestational age was assessed using the Dubowitz score. Thereafter, trained village monitors visited each mother-infant pair every fortnight for clinical observation and to administer a questionnaire on disease symptoms and any medication received. Monthly capillary blood samples were taken by finger or heel prick from infants for determination of malaria parasitemia and hemoglobin levels. Aliquots of peripheral blood were stored in liquid nitrogen and used as a source of DNA. Only children from singleton births and the first child of mothers who contributed more than one birth to the cohort were included in the study. There were 1,247 infants who met this criterion in the cohort, and DNA samples were available from 1,077 infants for genetic analysis. Genomic DNA was obtained from whole blood using the Puregene and Capture column DNA extraction kits (Gentra Systems, Minneapolis, MN) as directed by the manufacturer. From 1,077 DNA samples, we were able to genotype 1,048 samples. Twenty-nine samples could not be polymerase chain reaction (PCR)–amplified.

The TNF –308 was typed as described by Wilson et al. [1992]. Briefly, primers AL1566 (5′-AGG CAA TAG GTT TTG AGG GCC AT-3′) and AL1567 (5′-TCC TCC CTG CTC CGA TTC CG-3′) were used to amplify a 107-bp region of the TNF-α promoter region spanning the polymorphic site. Fifty to 100 ng of DNA was amplified in a PCR mixture containing 0.2 μ*M* of each primer, 1 × concentration of PCR buffer (PE Applied Biosystems, Foster City, CA, 1.25 U Taq polymerase (PE Applied Biosystems), and 200 μ*M* of each deoxyribonucleoside triphosphate (PE Applied Biosystems), in a final volume of 100 μL. PCR cycling conditions were as described by Wilson et al. [1992]. The amplified DNA fragment appears as a 107-bp band, which, when treated with *N*coI restriction enzyme, produces an 87- and a 20-bp band in the presence of *TNF1* but is undigested and remains a 107-bp band in the presence of *TNF2*.

Data were analyzed using univariate and multivariate Poisson regression models to determine the relationship between TNF –308 variants and morbidity. Survival analysis (Cox proportional hazard regression) was used to determine the relationship between *TNF2* and mortality. Covariates for statistical control included mother's survival (dead or alive) and educational level (<5 or ≥5 years), and the child's gestational age (<37 or ≥37 weeks), birth weight (<2,500 or ≥2,500 g), and gender. Prematurity and low birth weight were defined as less than 37 weeks' gestation and less than 2,500 g, respectively. Severe anemia was defined as hemoglobin level <6 g/dL and severe malarial anemia was defined as hemoglobin level <6 g/dL with a *P. falciparum* parasitemia of >10,000 parasites/μL of blood. These regression models were fit using the SAS statistical package (SAS Institute, Cary, NC) and Epi Info 6 (Centers for Disease Control, Atlanta, GA) was used to calculate risk ratios and *P*-

values. The statistical differences in the mean birth weights were determined using Tukey test for multiple comparisons.

RESULTS

We genotyped 1,048 DNA samples for the TNF –308 polymorphism. The gene frequencies of the *TNF1* and *TNF2* alleles were 0.90 and 0.10, respectively. The frequencies of different TNF –308 genotypes in this population are shown in Table I.

Our data analysis showed that the *TNF2* homozygote genotype is significantly associated with pre-term birth when compared with *TNF1* homozygotes (RR 7.3, 95% CI 2.85–18.9) and *TNF1/2* heterozygotes (RR 6.7, 95% CI 2.0–23.0) (Table I). The mean birth weight was lower among pre-term babies when compared with babies born at term; however, the difference was significantly different only for *TNF1* homozygotes ($P = 0.001$) and *TNF2* homozygotes ($P = 0.004$) but not for *TNF1/2* heterozygotes ($P = 0.12$). Among babies born pre-term, *TNF2* homozygotes had the lowest mean birth weights, but the difference was not significantly different when compared with *TNF1* homozygotes ($P = 0.79$) or *TNF1/2* heterozygotes ($P = 0.80$). The risk of being born with low birth weight was also higher for *TNF2* homozygotes when compared with *TNF1* homozygotes, but the difference was not significant (Table I). We also compared the risk of intrauterine growth retardation (IUGR) among the three TNF-α promoter variants but did not identify any (Table I).

TABLE I. The Frequency of TNF –308 Genotypes and Their Association With Birth Outcomes

Outcomes	*TNF1*	*TNF1/2*	*TNF2*
Genotype distribution	869/1,048	160/1,048	19/1,048
Normal gestation[a] (≥37 wk of gestation)	800 (97.0%)	147 (96.7%)	14 (77.7%)
Short gestation (<37 wk of gestation)	25 (3.0%)	5 (3.3%)	4 (22.2%)[b]
Mean birth weight of babies born at normal gestational age	3,106 ± 461 g[c] n = 769 (95% CI 3,073–3,139)	3,097 ± 467 g n = 143 (95% CI 3,020–3174)	3,124 ± 525 g n = 14 (95% CI 2,821–3,428)
Mean birth weight of babies born at short gestational age	2,484 ± 608 n = 24 (95% CI 2,228–2742)	2,560 ± 460 n = 5 (95% CI 1,988–3,132)	2,160 ± 768[d] n = 4 (95% CI 936–3,383)
No. of low birth weight children (observed/total)	69/819 (8.4%)	13/152 (8.6%)	3/19 (15.8%)[e] RR 1.87 (95% CI 0.6–6)
No. of IUGR children (observed/total)	152/742 (20.5%)	27/130 (20.8%)	5/18 (27.8%)[f] RR 1.36 (95% CI 0.6–3)

[a]We excluded 53 children whose gestational age was not available.
[b]*TNF2* versus *TNF1,* RR 7.3 (95% CI 2.85–18.9) $P = 0.002$, and *TNF2* versus *TNF1/2*, RR 6.7 (95% CI 2–23), $P = 0.008$.
[c]The mean birthweight ± standard deviation is given. n represents the number of children included in the analysis.
[d]The difference in the mean birthweight was not statistically different among babies born with short gestational age, i.e., between *TNF2* vs. *TNF1* ($P = 0.79$) or *TNF2* vs. *TNF1/2* ($P = 0.80$).
[e]No significant difference.
[f]No significant difference.

Survival analysis was performed to determine whether the *TNF2* allele was associated with mortality. Using multivariate analyses, which showed a strong interaction between gestational age and *TNF2*, we found that the *TNF2* allele was associated with increased mortality among children born prematurely (RR 7.47, 95% CI 2.36–23.6) (Fig. 1A). Among premature infants, the risk of childhood death was higher for *TNF2* homozygotes (*TNF2* homozygotes versus *TNF1* homozygotes: RR 70.9, 95% CI 18.2–277.2) than for *TNF1/2* heterozygotes (*TNF1/2* heterozygotes versus *TNF1* homozygotes: RR 3.43, 95% CI 0.82–14.36). There was no association of the *TNF2* allele with mortality among children born at term (≥37 weeks) (RR 0.86, 95% CI 0.58–1.27) (Fig. 1B).

In this study area, *P. falciparum* malaria accounts for a significant portion of mortality among children younger than 2 years. Therefore, we compared common manifestations of malaria morbidity among the TNF –308 genotypes (Table II). Potential confounding by gender, mother's education level, gestational age, birth weight, and the mother's survival status were assessed. Our data showed that *TNF2* was marginally associated with a higher incidence of high-density (>10,000/mL) *P. falciparum* blood infections (RR 1.11, 95% CI 1.00–1.24, $P = 0.043$). This risk was similar for *TNF2* homozygotes and *TNF1/2* heterozygotes (data not shown). Among low birth weight children there was a significant association of the *TNF2* allele with increased frequency of severe anemia (hemoglobin <6 g/dL) episodes (RR 2.16, 95% CI 1.17–4.01) as well as severe anemia episodes with positive *P. falciparum* parasitemia (RR 2.16, 95% CI 1.11–4.19). However, the increased risk associated with severe malaria anemia (hemoglobin <6 g/dL with >10,000 *P. falciparum* parasites/μL of blood) (RR 1.99, 95% CI 0.89–4.46) among children with *TNF2* was not significant (Table II).

DISCUSSION

Using a community-based longitudinal study, we have shown that *TNF2* homozygosity is associated with premature birth, and among those born prematurely, the *TNF2* allele is associated with increased mortality. Previous reports showed that high levels of TNF-α in amniotic fluid are associated with pre-term delivery [Shaarawy and Nagui, 1997; Arntzen et al., 1998; Romero et al., 1998; Wenstrom et al., 1998]. One of the mechanisms by which higher levels of TNF-α could accelerate pre-term labor is through up-regulation of cyclooxygenase (COX)-2 enzyme–mediated prostaglandin production [Perkins and Kniss, 1997; Swaisgood et al., 1997]. In addition, TNF-α is known to enhance the production of matrix metalloproteinases that can degrade collagen in fetal membranes and maternal tissues [So et al., 1992] and may lead to premature rupture of the membranes and cervical dilation [Roberts et al., 1999]. This interpretation is based on the assumption that *TNF2* is associated with increased TNF-α production as shown previously [Conway et al., 1997; Wilson et al., 1997; Louis et al., 1998; Warzocha et al., 1998].

This is the first study with a large enough sample size to show that *TNF2* in the fetus is associated with pre-term birth. Because this association was found only among *TNF2* homozygotes, by inference, the mothers of these children must have carried at least one *TNF2* allele. In a previous study, Dizon-Towson et al. [1997] observed that *TNF2* in neonates and mothers was not significantly associated with pre-term birth.

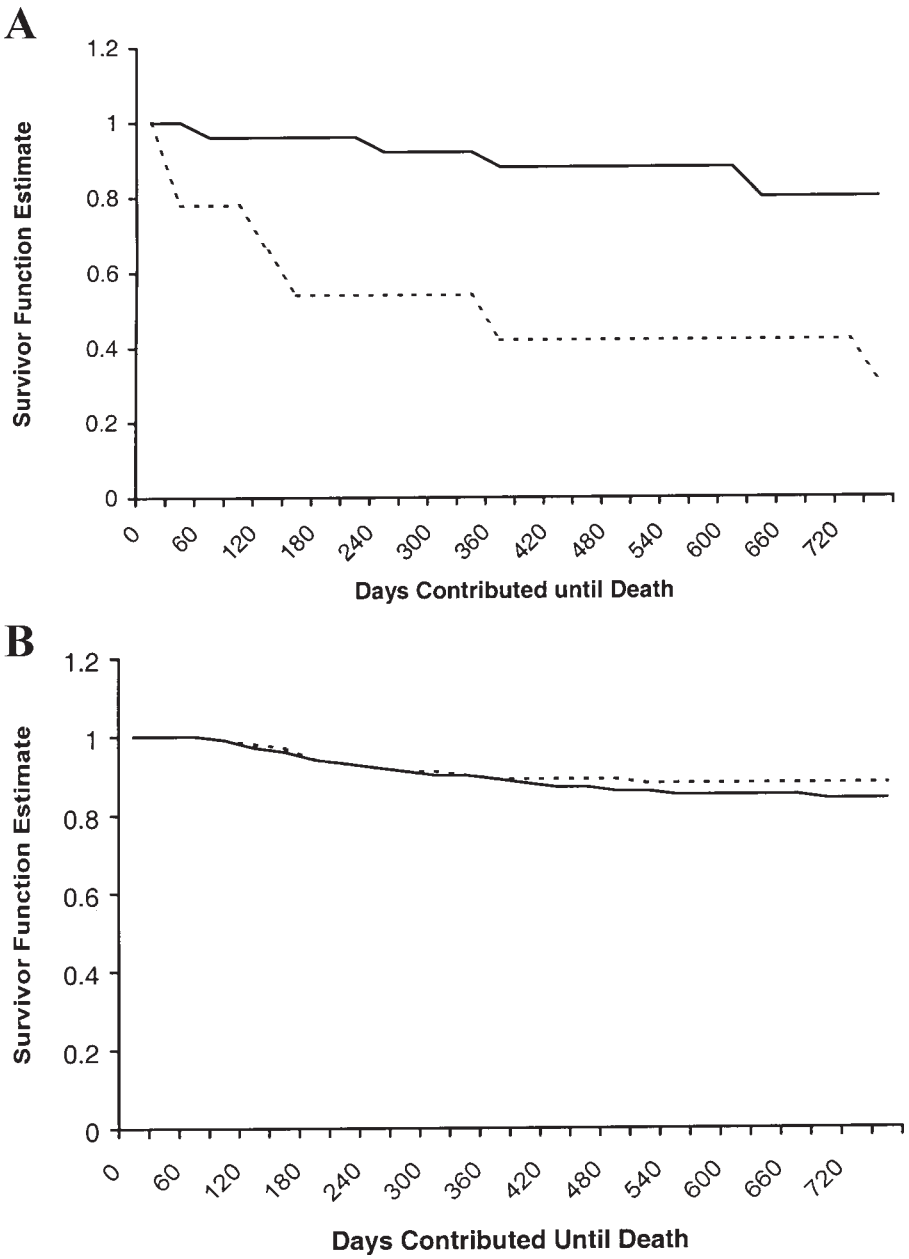

Fig. 1. **A:** Survival curves based on TNF-α –308 genotype for children born pre-term. Children with *TNF2* allele (broken line) had a higher risk for mortality than *TNF1* homozygote children (solid line) (RR 7.47, 95% CI 2.36–23.6). Twenty-five *TNF1* homozygote and nine *TNF2* (five heterozygotes and four homozygotes) children were included in the analysis. Twenty of 25 *TNF1* homozygotes, two of five *TNF2* heterozygotes, and zero of four homozygotes survived. **B:** Survival curves for children born at term show no difference in the survival rate between *TNF1* homozygote and *TNF2*-positive children (RR 0.86, 95% CI 0.58–1.27). Seven hundred eighty-eight *TNF1* homozygotes and 157 *TNF2* (144 heterozygotes, 13 homozygotes) were used in the analysis; 621 *TNF1* homozygotes and 128 *TNF2* homozygotes and heterozygotes survived.

TABLE II. Comparison of Malaria Morbidity Episodes Between *TNF1/1* Homozygotes and the Combination of *TNF2/2* Homozygotes and *TNF1/2* Heterozygotes

Morbidity	*TNF1/2 + TNF2/2* vs. *TNF1/1* (RR, 95% CI)
High density parasitemia (>10,000/μL)[a]	1.11, 1.00–1.24[b]
Any severe anemia (Hb <6 g/dL)	0.94, 0.74–1.88 (normal birth weight children) 2.16, 1.17–4.01 (low birth weight children)[b]
Severe anemia (Hb <6 g/dL) with any level of *P. falciparum* parasitemia	1.00, 0.79–1.29 (normal birth weight children) 2.16, 1.11–4.19 (low birth weight children)[b]
Severe malaria anemia (Hb <6 g/dL + parasitemia >10,000/μL)	1.05, 0.76–1.46 (normal birth weight children) 1.99, 0.89–4.46 (low birth weight children)

[a]The difference in the high-density parasitemia between individual genotypes, i.e., *TNF2* homozygotes, did not have any higher risk than *TNF1/2* heterozygotes when compared with *TNF1* homozygotes for the risk of high-density malaria parasitemia.
[b]Significant difference.
Hb, hemoglobin.

Conversely, Roberts et al. [1999] reported that the maternal *TNF2* allele was significantly associated with pre-term birth due to premature rupture of membranes and not with idiopathic pre-term delivery (another category of pre-term birth due to spontaneous contractions without any known antecedent pathology). Because premature rupture of membranes is associated with an infectious etiology in many cases, the findings of Roberts et al. [1999] and our results are consistent with the hypothesis that the increased TNF-α production due to the *TNF2* allele could have contributed to pre-term birth.

In this study, *TNF2* homozygosity was associated with a twofold increased risk of low birth weight. Although this increased risk was not a significant one, this observation is consistent with the fact that often pre-term birth is associated with a low birth weight. Overall, the lack of statistically significant difference in the mean birth weights and IUGR between the three TNF –308 genotypes suggests that *TNF2* is an independent risk factor for pre-term birth.

Our study shows for the first time that the *TNF2* allele is associated with all-cause mortality among infants born prematurely. The risk of mortality was much higher for *TNF2* homozygotes than for heterozygotes. In the Gambian study [McGuire et al., 1994], the *TNF2* homozygotes but not *TNF1/2* heterozygotes had a higher risk for cerebral malaria and its associated mortality. In other studies, *TNF2* has been found to be a risk factor for mortality due to meningococcal disease [Nadel et al., 1996] and septic shock [Mira et al., 1999]. The most common childhood illnesses in this study area are malaria, upper respiratory infection, gastrointestinal infections, and pneumonia. Because we did not have data on disease-specific mortality in this cohort, it was not possible to link *TNF2* associated deaths with any particular cause. Although in the Gambian study, the *TNF2* genotype was associated with an increased risk of mortality among children with cerebral malaria, we believe that such a risk is most probably not the cause of *TNF2*-associated deaths in this study due to the following reasons. First, cerebral malaria is rare in our study population in western Kenya [Snow et al., 1997] and, second, most of the deaths among the premature infants with *TNF2* occurred in the first 6 months of life, a time period during which cerebral malaria rarely occurs in children living in endemic areas.

In previous studies conducted in areas with seasonal malaria transmission, it

had been reported that *TNF2* was not associated with severe malaria anemia [McGuire et al., 1994, 1999]. On the contrary, in this study, we found that *TNF2* was associated with severe anemia among low birth weight children. It is also important to point out that the lack of statistical significance between *TNF2* and severe malarial anemia episodes could be simply due to the small sample size because there was a definite trend toward the increased relative risk (RR 1.99, 95% CI 0.89–4.46). In another study conducted in a holoendemic malaria transmission area in Tanzania [Stirnadel et al., 1999], infants with *TNF2* had higher but statistically insignificant mean *P. falciparum* parasite densities when compared with *TNF1*. Consistent with the Tanzanian study, we also found that *TNF2* was only marginally associated with a risk of high density *P. falciparum* parasitemia. Our estimates showed that less than 2% of the high-density parasitemia episodes could be attributed to the *TNF2* allele (attributable risk 0.019). Overall, the results from the morbidity analysis did not provide any evidence to suggest malaria as the definitive cause of the association of *TNF2* with mortality among pre-term babies. Caution, however, is needed in the interpretation of these observations because all children with positive blood films were treated with antimalarial drugs. The drug intervention could potentially lead to an underestimation of the association of *TNF2* with severe malarial anemia.

It is not clear why *TNF2* is associated with infant mortality among infants born pre-term. Prenatal exposure to TNF-α has been suggested to be a risk factor for respiratory distress syndrome [Speer, 1999]. It is possible that premature children born with *TNF2* may be at a high risk of developing life-threatening complications such as pneumonia, septic shock, or other complications of unknown etiology, especially when they have respiratory and or other organ systems that are not fully developed at birth. This hypothesis is consistent with our observation that *TNF2* homozygotes had a several-fold increase in the risk of death compared with *TNF1/2* heterozygotes. Recently, Mira et al. [1999] demonstrated that *TNF2* individuals were more susceptible to septic shock and were also more likely to die from it. In Sri Lanka, *TNF2* was found to be associated with severe complications resulting from malaria as well as impaired consciousness associated with bacterial and viral infections [Wattavidanage et al., 1999]. Clearly, the *TNF2* allele seems to be associated with severe disease caused by a variety of infectious agents, and it is plausible that abnormal levels of TNF-α could trigger a pathway that eventually leads to death. Further studies are required to test such a hypothesis.

Despite the observed association, *TNF2* may be only causally associated with mortality and that other closely linked genetic factors may play a role. The *TNF2* allele, which is located in the MHC class III region, is in linkage disequilibrium with some HLA class I and II antigens [Wilson et al., 1993]. We did not determine this linkage in our study; however, previous studies showed that *TNF2*-associated deaths among cerebral malaria [McGuire et al., 1994] and septic shock patients [Mira et al., 1999] were independent of linked HLA alleles. There are at least two other single nucleotide polymorphisms in the TNF-α gene promoter region. The TNF-238_A allele was found to be associated with severe malaria anemia [McGuire et al., 1999] and TNF-376_A was associated with cerebral malaria [Knight et al., 1999]. However, *TNF2* is not in linkage with any of these two polymorphisms [Knight et al., 1999; McGuire et al., 1999].

In conclusion, this study shows that *TNF2* homozygosity is associated with

pre-term birth and the *TNF2* allele is a risk factor for infant mortality among those born prematurely. A marginal association of *TNF2* with high-density *P. falciparum* parasitemia and severe anemia was also found. Further studies are needed to fully understand the mechanisms underlying these risks in infancy and possibly in the unborn child.

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